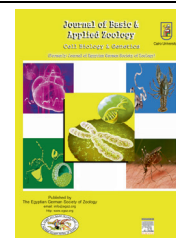




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Improvement in beta-islets of Langerhans in alloxan-induced diabetic rats by erythropoietin and spirulina

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Histology;
IHC;
Rats

Abstract The present study was undertaken to assess the effect of erythropoietin (EPO) and/or spirulina to treat alloxanized-diabetic rats. Eighty male albino rats were equally divided into eight groups; Group I: Normal control rats, Group II: Non-diabetic rats treated with EPO (40 U/kg) injected subcutaneously three times weekly for 3 weeks, Group III: Non-diabetic rats administered orally with spirulina (2 g/kg/d) for 21 days, Group IV: Non-diabetic rats treated by EPO (40 U/kg) together with spirulina (2 g/kg/d) as mentioned in groups II & III, Group V: Alloxanized-diabetic rats. Group VI: Diabetic rats treated with EPO (40 U/kg) as in group II, Group VII: Diabetic rats administered with spirulina (2 g/kg/d) as in group III, Group VIII: Diabetic rats were given with EPO (40 U/kg) and spirulina (2 g/kg/d) as in group IV. Diabetic rat group showed a significant increase in glucose and NO; and a significant decrease in insulin, SOD and CAT levels. Diabetic rats treated with EPO or/and spirulina recorded a significant decrease in the glucose and NO levels; and a significant increase in insulin, SOD and CAT levels when compared with the diabetic group. Histopathologically, diabetic rats treated with EPO or spirulina showed a slight improvement of pancreatic islets and acinar cells, diabetic rats treated with EPO & spirulina together showed an obvious recovery to approximately normal status. IHC, the expression of insulin producing cells (β -cells) of diabetic rats was improved in the three treatment groups with a lesser affinity for EPO than spirulina while with both together showed marked recovery into normal status. In conclusion, all the changes were minimized in spirulina administered group more than EPO group, however, the co-treatment of EPO and spirulina exerted stronger anti-hyperglycemic effects than treatment with each agent alone.

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Introduction

Diabetes mellitus is a universal metabolic disorder characterized by hyperglycemia, hyperlipidemia, hyperaminoacidemia

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and hypoinsulinemia that leads to reduction in both insulin secretion and insulin action (Altan, 2003). There is always a greater risk of all manifestations of atherosclerosis along with diabetes mellitus (Khuwaja et al., 2004), as well as association with a reduced quality of life and increase in risk factors of mortality and morbidity (Shaw et al., 2009). Type 2 diabetes mellitus is a chronic disorder of insulin insufficiency resulting in the dysregulation of glucose homeostasis, hyperglycemia and vascular complications. Diabetes has distinct pathogenic insufficient functional pancreatic β -cell mass that is required to maintain euglycemia (Kahn, 2003; Rhodes, 2005). Thus, one of the overarching goals in the treatment of diabetes is the preservation and growth of β -cells.

Erythropoietin (EPO), is a glycoprotein hormone with a molecular mass of 30.4 kD that controls erythropoiesis, it is produced by interstitial fibroblasts in the kidney in close association with peritubular capillary and tubular epithelial cells (Obara et al., 2008). It is also produced in perisinusoidal cells in the liver. While liver production predominates in the fetal and perinatal period, renal production is predominant during adulthood. EPO is the hormone that regulates red blood cell production. It also has other known biological functions (Hand and Brines, 2011; McGee et al., 2012). For example, it plays an important role in the brain's response to neuronal injury (Siren et al., 2001). EPO is also involved in the wound healing process (Haroon et al., 2003).

Recent studies have shown that the EPO protects against diabetes through direct effects on pancreatic cells. The EPO receptor (EPO-R) is present in nonerythroid tissues, including the pancreatic islets of human and rodents (Fenjves et al., 2003; Choi et al., 2010). In particular, several studies have shown the efficacy of EPO in providing cytoprotection in experimental models of tissue injury (Brines and Cerami, 2006).

EPO overexpression in human pancreatic islets has been shown to prevent cytokine-induced cell death (Fenjves et al., 2004). EPO deficiency and a higher incidence of anemia have been shown in individuals with diabetes, suggesting potential beneficial effects of EPO in the setting of diabetes (McGill and Bell, 2006; Thomas, 2006). EPO clinical trial for non-diabetic individuals with chronic renal failure was associated with a significant increase in the incidence of hypoglycemia which raises the intriguing possibility of a direct effect of EPO on pancreatic β -cells (Drüeke et al., 2006).

EPO-R belongs to the cytokine class I receptor superfamily and utilizes a similar signal transduction pathway as the receptors for growth hormone and prolactin, knockouts of these show defects in β -cell mass and function (Freemark et al., 2002; Liu et al., 2004). Collectively, these data raise the possibility that EPO signaling may have significant biological effects on β -cells and thus may be relevant to diabetes (Brines and Cerami, 2006; Choi et al., 2010).

Spirulina, refers to the dried biomass of the cyanobacterium, *Arthrospira platensis*, and is a whole product of biological origin. Spirulina is a name used to describe mainly two species of Cyanobacteria, *A. platensis* and *Arthrospira maxima* that are commonly used as food and as dietary supplement (Mühling et al., 2006). The number of research articles discussing the beneficial effects of spirulina is increasing every year. Spirulina is rich in proteins, carbohydrates,

polyunsaturated fatty acids, sterols and some more vital elements such as calcium, iron, zinc, magnesium, manganese and selenium. It is a natural source of vitamin B12, vitamin E, ascorbic acid, tocopherols and a whole spectrum of natural mixed carotene and xanthophylls phytopigments.

Some of the early health effects of spirulina were in its role in diabetes management and its significant plasma triglycerides reduction effects (total- and LDL-cholesterol), blood pressure lowering, improving the antioxidant status, as well as inflammatory effects (Eun et al., 2008). Recent reports note the importance of spirulina for its immunomodulatory, anti fatigue, radio protective and antioxidant effects particularly on the biochemical parameters such as SOD and CAT levels (Mendiola et al., 2010). Spirulina protects against diabetes through direct effects on pancreatic β -cells (Khursheed et al., 2012). Recent studies have shown an insulin-like protein extracted from spirulina to have the same molecular mass, immunoreactivity and retention time, detected by reversed-phase chromatography (RP-HPLC), to be similar to that of the bovine insulin (Anwer et al., 2012).

The present study aimed to assess the impact of treatment with erythropoietin and the natural extract of the marine algae (spirulina) separately or in combination on the physiological and histopathological parameters of experimentally-induced diabetes in rats.

Materials and methods

Animals and housing conditions

Eighty male albino rats (*Rattus rattus*) weighing 150 ± 5 g were used in the current study. They were obtained from the Breeding Unit of the Egyptian Organization for Vaccine and Biological Preparation, Cairo, Egypt. All rats were kept under the same environmental conditions for 2 weeks before the study. The animals were fed *ad Libitum* with a standard pellet diet and allowed free access of water and they were housed in metal cages in a well-ventilated animal room. All protocols and procedures adopted for the present investigation were in accordance with the approval of the Institutional Animal Ethics Committee of National Research Center and in accordance with recommendation of the proper care and use of laboratory animals.

Induction of diabetes mellitus in rats

The diabetes was induced in the animals by three intraperitoneal (i.p.) injections of alloxan monohydrate (Sigma Aldrich, USA) dissolved in acetate buffered-saline (Merck). The 1st dose was at a dose of 150 mg/kg as recommended by Bromme et al. (2000). The 2nd dose was at a 100 mg/kg of alloxan after 2 days. Finally, the 3rd dose of alloxan (100 mg/kg) was applied 5 days after the 2nd one. Note, the 2nd and 3rd injections were used to ensure the insult of diabetes through the experimental duration.

The rats were fasted overnight, collection of blood samples and sera glucose determination were drawn from their tail tips. Sera glucose estimation was done by one touch electronic glycometer using glucose test strips, and the glucose level more than 250 mg/dl was used in the present study.

EPO and spirulina treatment

EPO in the form of EPREX, (rHuEPO, Epoetin alfa) was obtained from (Sigma Aldrich, USA). Rats were injected subcutaneously (s.c.) with EPO (40 U/kg bw), three times weekly on alternating days for 3 weeks. Spirulina (International University for Vital Energy I.U.V.E, Egypt) was given to rats daily by intragastric gavage at a dose of 2 g/kg/d for 21 days.

Experimental design

Eighty male rats were equally divided into eight groups as follows:

Group I: Non-diabetic normal control rats daily injected with 0.1 ml saline solution.

Group II: Non-diabetic rats treated s.c. with EPO (40 U/kg bw) three times weekly on alternating days, for 3 weeks.

Group III: Non-diabetic rats administered orally with 2 g/kg/d spirulina for 21 days.

Group IV: Non-diabetic rats given EPO (40 U/kg bw) and spirulina (2 g/kg/d) as mentioned in groups II & III.

Group V: Alloxanized-diabetic rats.

Group VI: Diabetic rats treated with EPO (40 U/kg bw) three times weekly on alternating days, for 3 weeks.

Group VII: Diabetic rats administered with 2 g/kg/d spirulina for 21 days.

Group VIII: Diabetic rats co-treated with EPO (40 U/kg bw) and spirulina (2 g/kg/d) as mentioned in groups II & III.

At the end of experiment, rats were fasted for 10 h and then killed under mild (diethyl ether) anesthesia by decapitation. Blood was collected by cardiac puncture and immediately analyzed. Blood was collected in two different tubes, i.e. one with anticoagulant, potassium oxalate and sodium fluoride for plasma and the other without anticoagulant for serum separation. Plasma and sera were separated by centrifugation. For clinical chemistry parameters, blood samples were kept at room temperature for 1 h, the plasma was taken for biochemical assays. A part of pancreatic tissues were stored at 22 °C until analyzed and the others were immediately fixed in 10% neutral formalin for histological and immunohistochemical studies.

Biochemical parameters

Biochemical parameters were measured using standard kits. Insulin was determined by the ALPCO. Rat Insulin ELISA is designed for the quantitative determination of insulin in rat sera and plasma (Finlay and Dillard, 2007), SOD EnzyChrom™ Superoxide Dismutase Assay Kit was used for SOD (Kuthan, 1986) NO QuantiChrom™ Nitric Oxide Assay Kit was used for NO (Ghigo, 2006), CAT Oxi Select Catalase Activity Assay Kit was used for CAT (Ames et al., 1993). Blood glucose estimation was done by one touch electronic glycometer using glucose test strips.

Histological and immunohistochemical (IHC) studies

For histological studies, pieces of pancreas were fixed in 10% neutral formalin, they were dehydrated through alcohols,

cleared in xylene and then embedded in paraffin. Paraffin sections were cut at 5 µm thick and were stained with hematoxylin and eosin “H&E” (Bancroft and Gamble, 2002) for histological observation.

IHC detection of pancreatic tissues with immunolocalization technique for anti-insulin monoclonal antibody was performed as previously described (Hsu et al., 1981). IHC reaction was carried out by using avidin biotin peroxidase method by Nova Castra Laboratories Ltd, UK. Endogenous peroxidase activity was inhibited by incubation with 0.3% H₂O₂ for 30 min. The sections were blocked with normal goat serum for 1 h to prevent non-specific binding followed by incubation with the primary insulin monoclonal antibody for 1 h at room temperature. The sections were incubated with the secondary antibody (biotinylated anti-mouse IgM) for 30 min. The sections were then incubated with ExtrAvidin (Sigma) for 45 min at 37 °C. Staining was visualized using diaminobenzidine (DAB, Sigma), then slides were washed and counterstained with hematoxylin, cleared, mounted and examined by light microscopy. Finally, the insulin secreting β-cells cytoplasmic sites of reaction were stained brown and nuclei stained blue.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS® software package version 20.0, USA. Data were analyzed using numbers and percentages ± S.D or S.E. For normally distributed data, comparisons between the eight studied groups were analyzed using *F*-test (ANOVA) and Post Hoc test (LSD). Significance was obtained at $P < 0.05$ or $P < 0.01$ or $p \leq 0.001$ levels.

Results

Physiological and biochemical observations

Effect of EPO or/and spirulina on glucose levels

As shown in Table 1, the data revealed that the treatment of rat regimes of EPO, spirulina or both together did not affect the plasma glucose levels as compared to the normal non-treated rats in group (1). On the other hand, a significant increase in the plasma glucose levels in alloxanized-diabetic rats (groups 5–8) was found comparable to the data from the non-diabetic rat groups (1–4). Treatment with EPO or spirulina or both together (groups 6–8) respectively to diabetic rats has significantly reduced the plasma glucose levels, particularly in group (8) which had a reduced glucose levels close to normal values.

Effect of EPO or/and spirulina on plasma insulin levels

Data represented in Table 2 showed that treatment of rats with EPO, spirulina or both in groups (2–4) respectively have significantly increased the plasma insulin levels as compared to normal controls in group (1). On the other hand, the non-treated diabetic rats in group (5) showed a significant decrease in the insulin levels as compared to the normal rats in group (1). Treatment of diabetic rats with EPO, spirulina or co-treatment (groups 6–8) respectively has exerted a significant increase in the plasma insulin levels as compared to control diabetic rats in group (5). The co-treatment with EPO and spirulina in group (8) exerted the most profound effect (Table 2).

Table 1 Effect of EPO, spirulina or both on plasma glucose levels (mg/dl).

Cases	Normal (G1)	Non-diabetic + EPO (G2)	Non-diabetic + spirulina (G3)	Non-diabetic EPO + spirulina (G4)	Control diabetic (G5)	Diabetic + EPO (G6)	Diabetic + spirulina (G7)	Diabetic EPO + spirulina (G8)
Mean \pm SD.	92.4 \pm 1.7	90.7 \pm 0.9	90.4 \pm 0.7	90.1 \pm 1.2	257.9 ^{a**} \pm 3.6	231.7 ^{a**b**} \pm 6.4	222.5 ^{a**b**} \pm 6.8	110.7 ^{a**b**} \pm 7.5

^a Significant vs. normal control group.^b Significant vs. control diabetic group.** Significant at $P < 0.01$.**Table 2** Effect of EPO or spirulina or both together on plasma insulin levels (μ /ml).

Cases	Normal (G1)	Non-diabetic + EPO (G2)	Non-diabetic + spirulina (G3)	Non-diabetic EPO + spirulina (G4)	Control diabetic (G5)	Diabetic + EPO (G6)	Diabetic + spirulina (G7)	Diabetic EPO + spirulina (G8)
Mean \pm SD.	14.88 \pm 49	15.73 ^{a*} \pm 0.75	16.24 ^{a**} \pm 0.65	16.48 ^{a**} \pm 0.42	4.84 ^{a**} \pm 0.28	6.61 ^{a**b**} \pm 0.44	6.78 ^{a**b**} \pm 0.13	14.19 ^{a*b**} \pm 1.31

^a Significant vs. normal control group.^b Significant vs. control diabetic group.* Significant at $P < 0.05$.** Significant at $P < 0.01$.

Table 3 Effect of EPO or/and spirulina on plasma SOD levels (U/mg).

Cases	Normal (G1)	Non-diabetic + EPO (G2)	Non-diabetic + spirulina (G3)	Non-diabetic EPO + spirulina (G4)	Control diabetic (G5)	Diabetic + EPO (G6)	Diabetic + spirulina (G7)	Diabetic EPO + spirulina (G8)
Mean \pm SD.	6.37 \pm 0.39	6.54 ^{a*} \pm 0.33	6.81 ^{a***} \pm 0.44	7.91 ^{a***} \pm 0.32	2.75 ^{a***} \pm 0.49	4.35 ^{a***b***} \pm 0.37	5.46 ^{a***b***} \pm 0.41	5.85 ^{a***b***} \pm 0.39

^a Significant vs. normal control group.^b Significant vs. control diabetic group.* Significant at $P < 0.05$.** Significant at $P < 0.01$.*** Significantly at $p < 0.001$.**Table 4** Effect of EPO or/and Spirulina on Plasma CAT Levels (nmol H₂O₂/s per g).

Cases	Normal (G1)	Non-diabetic + EPO (G2)	Non-diabetic + spirulina (G3)	Non-diabetic EPO + spirulina (G4)	Control diabetic (G5)	Diabetic + EPO (G6)	Diabetic + spirulina (G7)	Diabetic EPO + spirulina (G8)
Mean \pm SD.	12.10 \pm 0.32	12.89 ^{a***} \pm 0.52	14.06 ^{a***} \pm 0.25	15.16 ^{a***} \pm 0.27	4.36 ^{a***} \pm 0.42	6.30 ^{a***b***} \pm 0.42	8.44 ^{a***b***} \pm 0.46	10.22 ^{a***b***} \pm 0.53

^a Significant vs. normal control group.^b Significant vs. control diabetic group.*** Significantly at $p < 0.001$.

Effect of EPO or/and spirulina on plasma SOD levels

As shown in Table 3, treatment of the non-diabetic rats in groups (2–4) respectively with EPO or/and spirulina have significantly increased the plasma SOD levels as compared to the normal control rats in group (1), with the levels in group (4) to be the highest. Moreover, all diabetic rats in groups (5–8) had significant lower plasma SOD levels as compared to the non-diabetic rats in groups (1–4). On the other hand, treatment of the diabetic rats in groups (6–8) with EPO or spirulina or together respectively, have significantly elevated the plasma SOD levels, particularly in group (8), as compared to the control diabetic rats in group (5).

Effect of EPO or/and spirulina on plasma CAT levels

Data in Table 4 showed that treatment of the non-diabetic rats in groups (2–4) respectively with EPO or/and spirulina have significantly increased the plasma CAT levels as compared to the normal control rats in group (1), with the levels in group (4) to be the highest. Moreover, all diabetic rats in groups (5–8) had a significant lower plasma CAT levels as compared to the non-diabetic rats in groups (1–4). The treatment of the diabetic rats in groups (6–8) with EPO or spirulina or both respectively, have significantly elevated the plasma CAT levels, particularly in group (8), as compared to the control diabetic rats in group (5).

Effect of EPO or/and spirulina on plasma NO levels

Data in Table 5 showed that treatment of the non-diabetic rats in groups (2–4) respectively with EPO or spirulina or both have significantly decreased the plasma NO levels as compared to the normal control rats in group (1), with the levels in group (4) to be the lowest. Moreover, all diabetic rats in groups (5–8) had significant higher plasma NO levels as compared to the non-diabetic rats in groups (1–4). On the other hand, treatment of the diabetic rats in groups (6–8) with EPO or spirulina or together respectively, have significantly decreased the plasma NO levels, particularly in group (8), as compared to the control diabetic rats in group (5).

Histological observations

The pancreatic sections of the normal control rats (non-diabetic rats group) stained with H&E showed normal architecture of the pancreatic acini and islet of Langerhans. The pancreatic acini consist of pyramidal cells containing basal rounded nuclei. The apical region of each pyramidal cell contains acidophilic granules (zymogenic granules), and the basal part is basophilic. The islets of Langerhans are scattered between the acini, they are rounded or oval in configuration and appeared faintly stained with H&E (Fig. 1). The pancreatic sections of the non-diabetic rats treated with EPO or spirulina or co-treated with both agents (groups 2–4) have also shown normal histological architecture (Figs. 2–4).

The sections of the pancreas of the alloxanized-diabetic rats (group 5) showed necrotic areas, vacuolation in islet cells and condensed fibers around many blood capillaries (Fig. 5a and b). In diabetic rats treated with EPO (group 6), the pancreatic sections showed slight improvement in the minimization of the vacuolated islets cells, reduction of fibers around the blood vessels (Fig. 6). Diabetic rats administered with spirulina

Table 5 Effect of EPO or/and Spirulina on Plasma NO Levels (nitrite/mg protein).

Cases	Normal (G1)	Non-diabetic + EPO (G2)	Non-diabetic + spirulina (G3)	Non-diabetic EPO + spirulina (G4)	Control diabetic (G5)	Diabetic + EPO (G6)	Diabetic + spirulina (G7)	Diabetic EPO + spirulina (G8)
Mean ± SD.	3.35 ± 0.32	1.60 ^{a***} ± 0.23	1.29 ^{a***b***} ± 0.20	0.81 ^{a***b***} ± 0.07	6.13 ^{a***b***} ± 0.47	5.26 ^{a***b***} ± 0.42	4.80 ^{a***b***} ± 0.50	4.21 ^{a***b***} ± 0.27

^a Significant vs. normal control group.

^b Significant vs. control diabetic group.

** Significant at $P < 0.01$.

*** Significantly at $p < 0.001$.

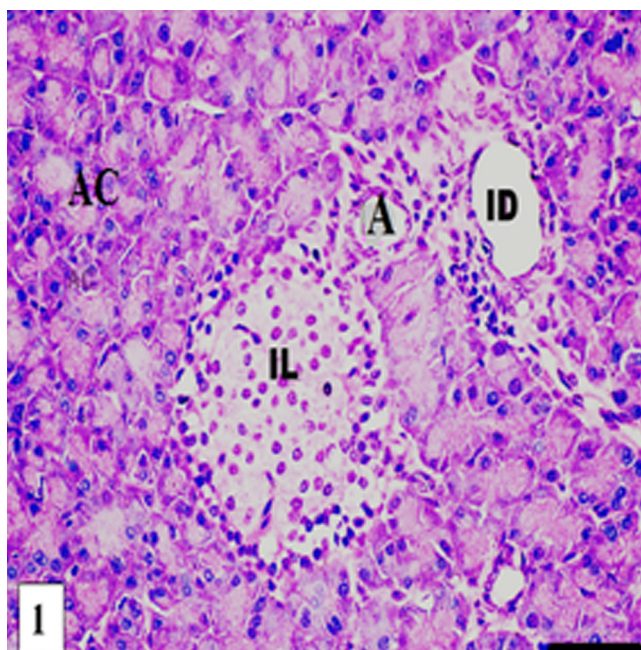


Figure 1 A photomicrograph of a pancreatic section of a normal control rat showing normal architecture of acini (AC), islets of Langerhans (IL), intercalated duct (ID) and pancreatic artery (A). H&E, scale bar = 6.25 μ m.

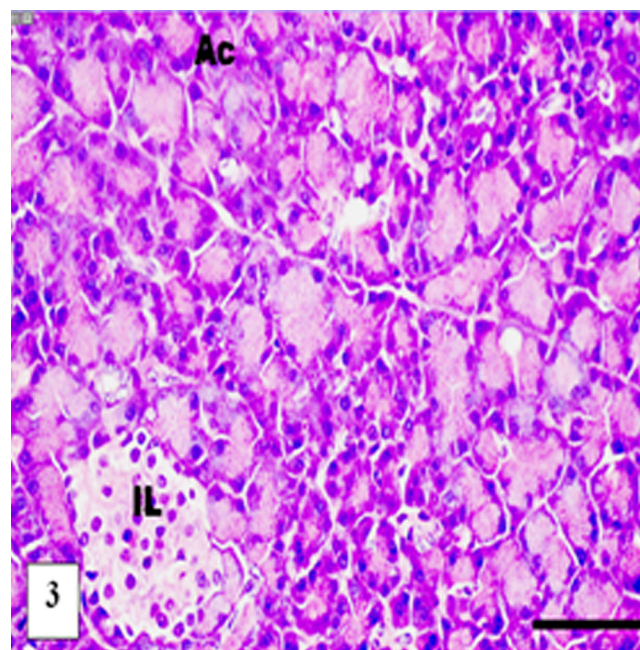


Figure 3 A photomicrograph of a pancreatic section of a non-diabetic rat administered with spirulina showing normal appearance of acini (AC) and islet of Langerhans (IL). H&E, scale bar = 6.25 μ m.

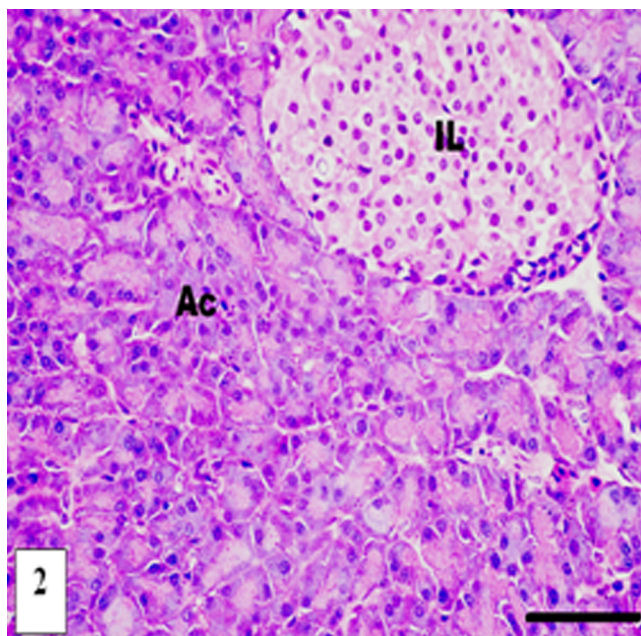


Figure 2 A photomicrograph of a pancreatic section of a non-diabetic rat treated with EPO showing normal appearance of acini (AC) and islet of Langerhans (IL). H&E, scale bar = 6.25 μ m.

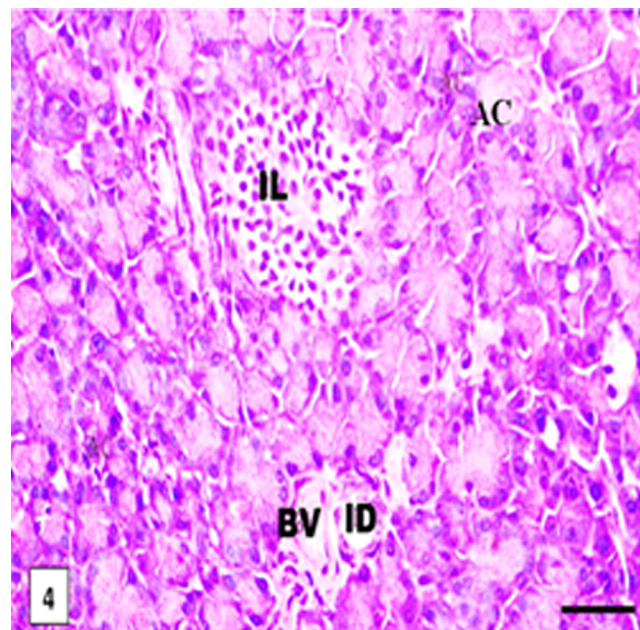


Figure 4 A photomicrograph of a pancreatic section of a rat given EPO + spirulina showing normal architecture of acini (AC), islets of Langerhans (IL), intercalated duct (ID) and blood vessels (BV). H&E, scale bar = 6.25 μ m.

demonstrated marked recovery of the pancreatic islets and acinar cells (Fig. 7). Diabetic rats treated with EPO and spirulina together showed an obvious recovery of pancreatic islet and acinar cells to almost normal structure and normal distribution of fibers surrounding the blood vessels (Fig. 8).

IHC observations

The pancreas of the normal control rats expressed normal strong immunoreactivity to the insulin secreting β -cells (Fig. 9). Similar normal results were expressed to β -cells in

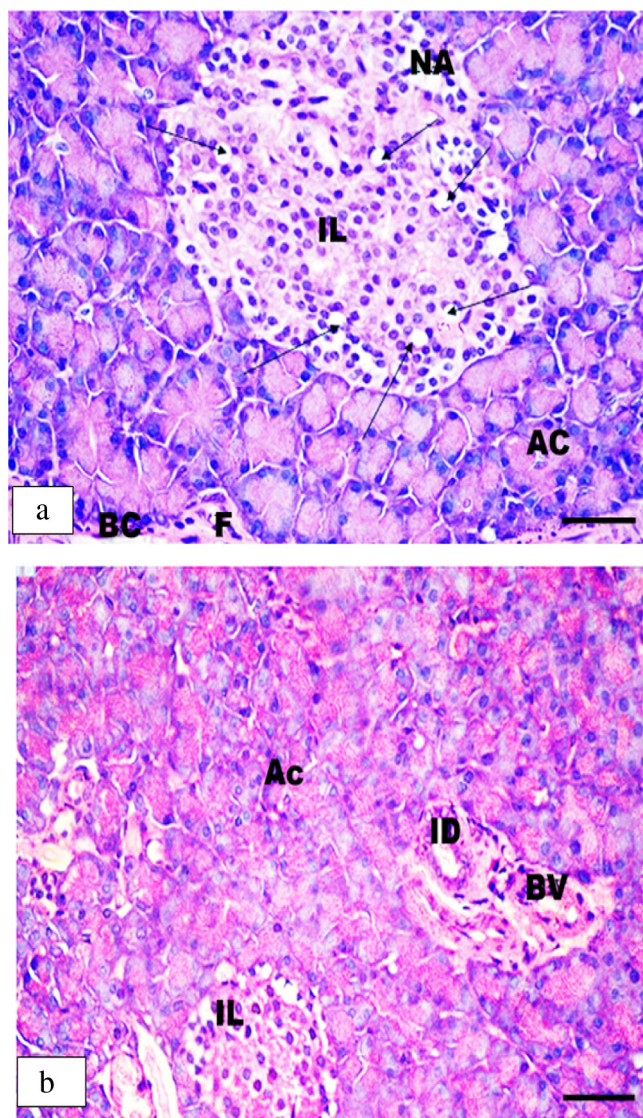


Figure 5 (a and b) A photomicrograph of a pancreatic section of alloxanized-diabetic rat showing: (a) necrotic areas (NA), vacuolated cells (arrows) in islet of Langerhans (IL), abnormal acinar cells (AC) and condensed fibers (F) around a blood capillary (BC); (b) vacuolated islet cells (IL), anomalous acinar cells (AC) and markedly accumulated fibers around pancreatic duct (ID) and blood vessel (BV). H&E, scale bar = 6.25 μ m.

the non-diabetic rats treated with EPO, spirulina or both together (data not shown). The alloxanized-diabetic rats showed less expression of insulin secreting β -cells with clear necrotic areas in islets of Langerhans (Fig. 10). The alloxanized-diabetic rats given with the three treatment regimes demonstrated an obvious increment in the expression of insulin secreting β -cells. Animals treated with EPO showed a less immunoreactivity (Fig. 11) in comparison to the diabetic rats administered with spirulina (Fig. 12). The diabetic rats treated with both EPO and spirulina together showed an obvious recovery as indicated by the increase in the expression of the insulin secreting β -cells (Fig. 13).

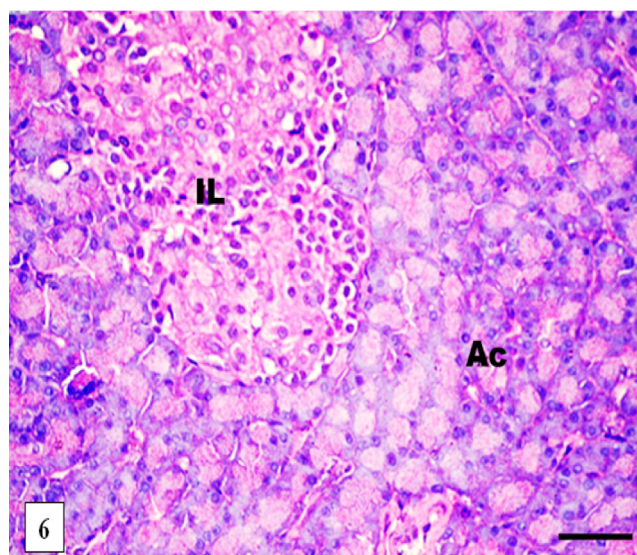


Figure 6 A photomicrograph of a pancreatic section of a diabetic rat treated with EPO showing the minimization of the vacuolated cells and a reduction of necrotic areas in islet of Langerhans (IL) and a recovery of most normal acinar cells (AC). H&E, scale bar = 6.25 μ m.

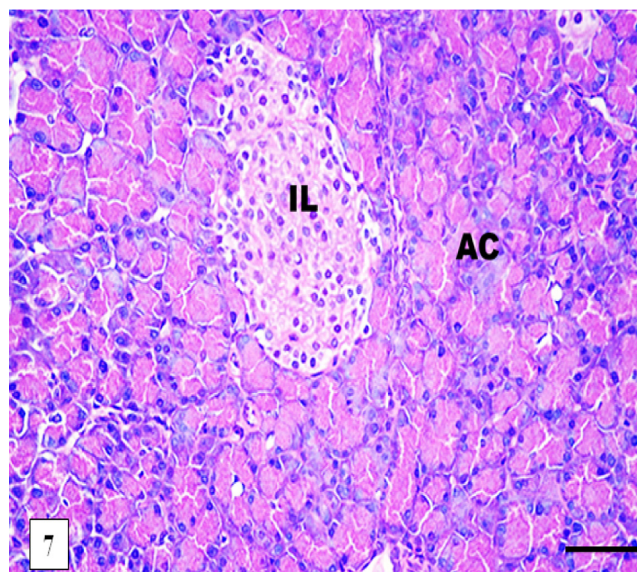


Figure 7 A photomicrograph of a pancreatic section of a diabetic rat administered with spirulina showing marked improvement of pancreatic islet (IL) and acinar cells (AC). H&E, scale bar = 6.25 μ m.

Discussion

Diabetes is a pancreatic β -cell defect, manifested by β -cell death and dysfunction. Alloxan is a diabetogenic agent which is widely used in studies of experimental diabetes because it selectively destroys the pancreatic β -cells of rats (El-Desouki, 2004) possibly through the mechanism of induction of free radical species (Szkudelski, 2001) and oxidative stress that

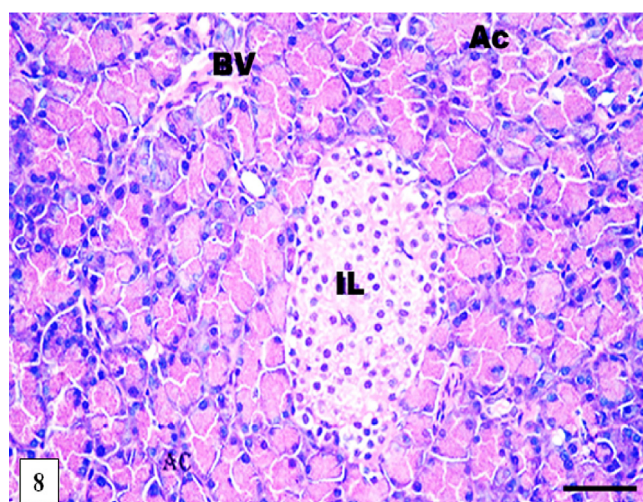


Figure 8 A photomicrograph of a pancreatic section of a diabetic rat given EPO and spirulina showing an obvious recovery of pancreatic islet (IL) and acinar (AC) cells to almost normal structure. See normal blood vessels (BV). H&E, scale bar = 6.25 μ m.

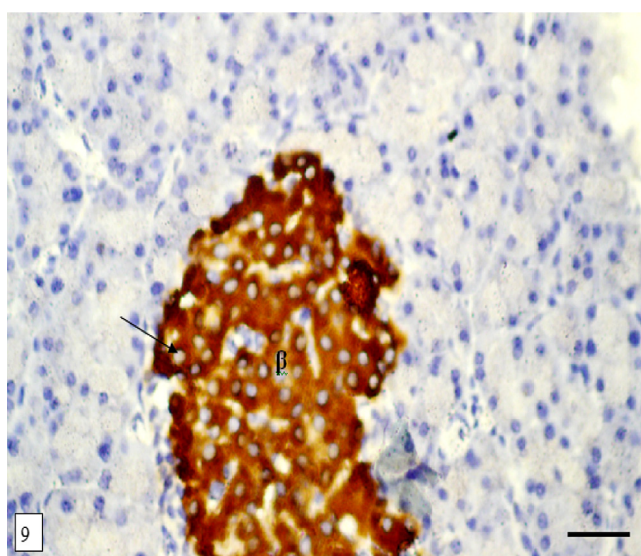


Figure 9 A photomicrograph of a pancreatic section of a control rat showing strong expression of insulin secreting β -cells (arrow). Anti-insulin immunostain, scale bar = 6.25 μ m.

impaired insulin secretion in type 2 diabetes (Robertson, 2006). In the recent study, the plasma glucose values were significantly increased and insulin levels were significantly decreased after induction of diabetes by alloxan in rats, and the treatment of diabetic rats with EPO (40 u/kg) or/and spirulina (2 g/kg/d) for 3 weeks successfully ameliorated the diabetic complications by declined glucose levels and enhanced again insulin levels reflecting a restoration of the pancreatic β -cells activity. These results were in agreement with the reports that have documented previously the effect of EPO on reduction blood glucose levels in human and experimental animals (Rasic-Milutinovic et al., 2008) and may result from an

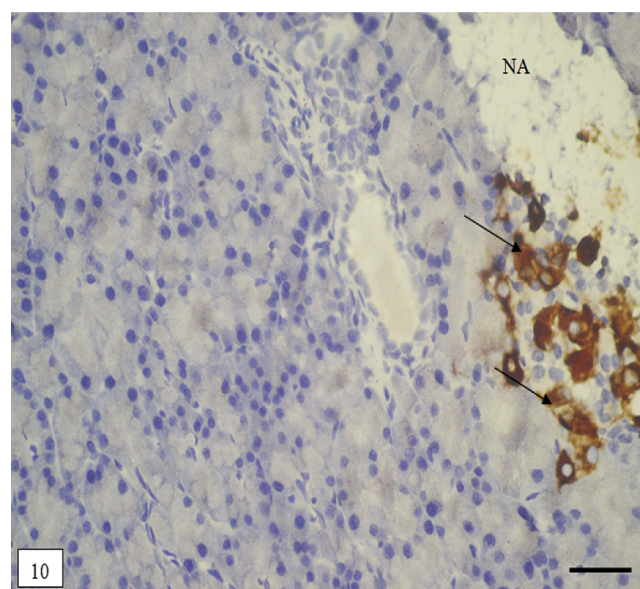


Figure 10 A photomicrograph of a pancreatic section of alloxanized-diabetic rat demonstrating a decrease expression of insulin secreting β -cells (arrows) and a clear necrotic area (NA) in an islet. Anti-insulin immunostain, scale bar = 6.25 μ m.

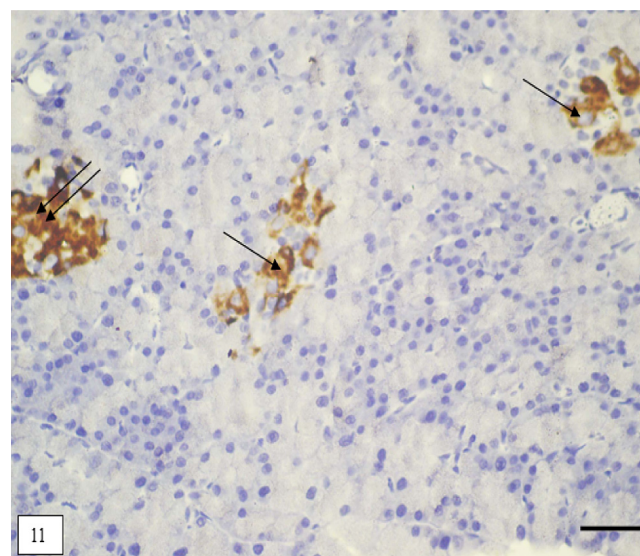


Figure 11 A photomicrograph of a pancreatic section of a diabetic rat treated with EPO showing a limit increase in the expression of insulin secreting β -cells in some islets (one arrow) and more expression in other islet (double arrow). Anti-insulin immunostain, scale bar = 6.25 μ m.

increase in the erythrocyte counts and their consequent uptake of glucose (Montel-Hagen et al., 2009).

EPO treatment has antiapoptotic, proliferative and angiogenic effects on the pancreatic islets. It inhibits apoptosis in β -cells during diabetes progression (Motoyama et al., 1995). Fenjves et al. (2003) recorded that EPO treatment ameliorated and reduced insulin levels to a great extent due to its effect directly on pancreatic β -cells. Similar results were recorded

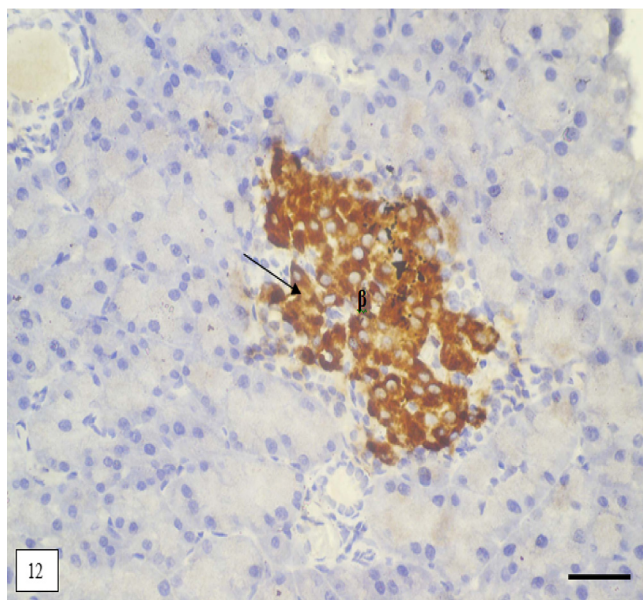


Figure 12 A photomicrograph of a pancreatic section of a diabetic rat administered with spirulina seeing marked improvement and an increase in the expression of insulin secreting β -cells (arrow). Anti-insulin immunostain, scale bar = 6.25 μ m.

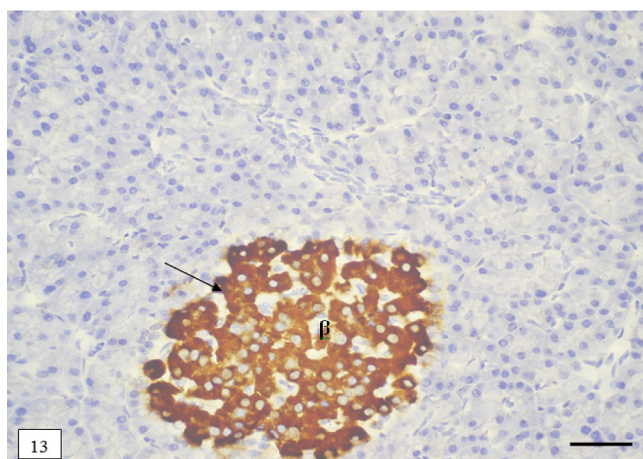


Figure 13 A photomicrograph of a pancreatic section of a diabetic rat given EPO and spirulina demonstrating a marked recovery of normal strong expression of insulin secreting β -cells (arrow). Anti-insulin immunostain, scale bar = 6.25 μ m.

by Marzo et al. (2008). However, Brines and Cerami (2006) demonstrated that the protective effect of EPO on the β -cells under diabetes conditions occurs largely through the promotion of β -cell growth and survival rather than through direct effects on β -cell function.

The present results showed that the possible mechanism by which spirulina brings about its antihyperglycemic action may be through potentiation of the pancreatic secretion of insulin from islet β -cells or due to enhancement of the blood glucose transport to the peripheral tissue (Layam and Reddy 2006).

The biochemical results in the present work demonstrated that the diabetic rats displayed a significant increment in NO levels while SOD and CAT were significantly declined. Many

studies suggested that the hyperglycemia has been associated with oxidative stress and increased levels of NO and reactive oxygen species (ROS) that have been proposed to induce insulin resistance (Chong et al., 2005). The author and his colleagues recorded that NO exerted a deleterious effect on β -cells through the inactivation of enzymes that are specifically protective against oxidative stress damage. Oxidative stress and NO pathways were related and seemed to modulate each other, leading to β -cells destruction in diabetes. The excess of NO production in the pancreas of diabetes involved an increase activity of nitric oxide synthase (NOS), whose level was higher in diabetic than in control tissue. The enhancement of NOS activity in diabetic tissues was the result of the induction of the Ca^{2+} -independent isoform. This agreed with McDaniel et al. (1996) who stated that β -cells, selectively destroyed in diabetes, seem to express the inducible isoform of NOS and to overproduce NO, which exerts deleterious effects on their function.

Also, high amounts of ROS have been shown to play a role in the development of diabetic complications as well as in a number of other disease states. ROS generated during metabolism can enter into reactions that, when uncontrolled, can affect certain processes leading to clinical manifestations. Oxidative stress induced by excessive production of superoxide and an imbalance in antioxidant enzymes (SOD & CAT) has been linked to the development of diabetic complications (Kesavulu et al., 2000).

The current results recorded that the treatment of EPO or/and spirulina to diabetic rats caused a significant decrease of the NO levels and increased in the antioxidant SOD and CAT values. Many authors reported that EPO may reflect another consideration for diabetic therapeutic strategies. Protection by EPO in a number of cellular systems can block apoptotic injury from a number of sources, such as reduced or absent oxygen tension, cytotoxicity and free radical exposure (Li et al., 2004, 2006). EPO treatment resulted in reduced lipid peroxidation and enhanced SOD, CAT, and other antioxidant activities. Moreover, EPO is a highly sialidated glycoprotein. It has been reported that mucin, a typical sialic acid containing high-molecular weight glycoprotein, is an anti-oxidant and that sialic acid is crucial for this activity (Ogasawara et al., 2007).

Concerning spirulina, many studies indicated the cyanobacterial proteins in spirulina are the molecules of high potency to work as antioxidants by scavenging peroxy, hydroxyl, peroxynitrite, superoxide radicals, and as inhibitors of lipid peroxidation (Bhat and Madyastha, 2001). Other studies suggested that spirulina contains several active ingredients, notably phycocyanin and β -carotene that have potent antioxidant and anti-inflammatory activities (Shih et al., 2009; Manconia et al., 2009).

The histological and IHC observations confirmed the biochemical data in the current study. The results showed that the diabetes caused changes in rat pancreatic tissue as islet vacuolization, degeneration and necrosis of β -cells, dilation of intercalated duct and infiltration of inflammatory cells. Moreover, the expression of insulin secreting cells (β -cells) by using monoclonal anti insulin markedly demonstrated the destruction and reduction of β -cells immunoreaction in diabetic rats. EPO or spirulina or co-treatment minimized and improved the changes associated with pancreatic rat diabetes and enhanced the expression of β -cells in the islets of

Langerhans, more improved in spirulina than in EPO rat groups, and the co-treatment of EPO and spirulina exerted stronger anti-hyperglycemic effects than treatment with each agent alone. Similar results have been reported by many authors; Marchetti et al. (2010) demonstrated the reduction of islet number and/or diminished beta-cell mass/volume in the pancreas of type 2 diabetes and morphological changes in several beta-cell organelles. Moreover, Patel et al. (2014) showed a decrease counting analysis in the number of pancreatic β -cells immunostain of obese-hyperglycemic mice that indicated the islets were losing the ability to secrete insulin efficiently (Deng et al., 2004).

EPO and spirulina have protective effects on pancreatic β -cell functions and may due to their powerful antioxidant properties. Supporting these findings was discussed above.

In conclusion, all the changes in the physiological parameters, histopathological and the degeneration of pancreatic β -cells immunoreactivity of alloxanized-diabetic rats were improved by spirulina more than by EPO, and the co-treatment of EPO and spirulina acts as hypoglycemic effect than the treatment with each separately.

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